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Received 5 January 1998;
accepted 12 August 1998

Binding of Proteins to Copolymers of Varying Hydrophobicity

Abstract: *Hydrophobic interactions between proteins and amphiphilic polyelectrolytes were studied by frontal analysis continuous capillary electrophoresis (Gao et al., Analytical Chemistry, 1997, Vol. 69, pp. 2945–2951). Binding isotherms were obtained for β-lactoglobulin and for bovine serum albumin interacting with a series of alternating copolymers of maleic acid and alkyl-vinyl ethers of varying hydrophobicity. Although binding between proteins and copolymers increases with increasing alkyl chain length, a minimum alkyl chain length of 3–4 methylenes is required for significant hydrophobic interactions to occur. These copolymers, like other polyamphiphiles, can form intrapolymer micelles, and the extent of such micellization decreases with increasing degree of carboxylate ionization. Binding results obtained at different pHs suggest that competition exists between intrapolymer micelle formation and protein–polymer hydrophobic interactions.* © 1999 John Wiley & Sons, Inc. Biopoly 49: 185–193, 1999

Keywords: *protein binding; copolymers; hydrophobicity; amphiphilic polyelectrolytes; isotherms; polyamphiphiles; intrapolymer micelle formation; protein–polymer interactions*

INTRODUCTION

The heterogeneity of protein surfaces leads to manifold types of interactions between proteins and other macromolecules. Thus, protein complexation with polymers in aqueous solution may be driven by hydrogen bonding,[1] hydrophobic interaction,[2] and electrostatic forces.[3] Hydrogen bonding can occur only under stringent conditions since it requires not only the presence of hydrogen-bond donors and acceptors, but also the spatial alignment of the donor–acceptor pairs. Consequently, electrostatic and hydrophobic interactions are more common factors in protein–polyelectrolyte complexation. Extensive efforts[4–6] have been made to study and model the former type of effects. However, the understanding of protein–polymer hydrophobic interactions has been hampered in part by the difficulty in quantifying these interactions. Nevertheless, the hydrophobic interaction between proteins and synthetic polymeric materials is an important phenomenon with useful applications. In reverse phase liquid chromatography (RPLC) and hydrophobic interaction chromatography (HIC), hydrophobic interactions between protein and stationary phase polymer are central to the protein retention mechanism. In aqueous colloidal solutions, hydropho-

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Biopolymers, Vol. 49, 185–193 (1999)
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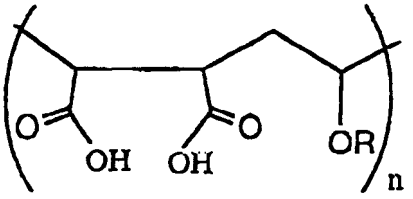
CCC 0006-3525/99/020185-09

bically modified water-soluble polymers are effective in stabilizing hydrophobic protein dispersions.[7,8]

Since the quantitative study of hydrophobic interactions in such systems is frequently complicated by the difficulty of resolving this effect from the others mentioned, hydrophobic interactions are commonly presumed to be ubiquitous even in the absence of concrete evidence. However, in some cases, hydrophobic contribution to protein–polymer interactions may be clearly evident. For example, Xia et al.[9] found that lysozyme interacts with pyrene-labeled sodium poly(2-acrylamido-2-methylpropanesulfonate) preferentially at pyrene sites due to hydrophobic interactions. Qualitatively, it is understood that the extent of hydrophobic interaction must be directly related to the hydrophobicity and spatial complementarity of each species involved in the macromolecular complexation, as shown by systematic studies[10–14] of interactions between proteins and homologous series of functionalized alkyl chains immobilized on agarose columns in HIC.[15] However, there has not been such a study for the hydrophobic interactions between proteins and amphiphilic polyelectrolytes in aqueous solution. Therefore, the main purpose of this work is to quantify these hydrophobic interactions by a systematic study of protein binding to a homologous series of amphiphilic polyelectrolytes. Such a study should shed light on procedures to evaluate the hydrophobic nature of a water-soluble polymer with regard to its complexation with proteins.

Many theoretical studies have previously addressed the role of protein hydrophobic bonding in tertiary structure and biological function.[16–19] Protein hydrophobicity has also been characterized in various ways. Waugh's *NPS* is a frequency of nonpolar side chains.[20] Fisher's p is the ratio of the volume occupied by polar residues to that occupied by the nonpolar residues.[21] Bigelow[22] proposed a parameter of average hydrophobicity $H\Phi_{ave}$, based on Tanford's free energies of transfer of amino acid side chains from organic environment to aqueous solution.[23] All these parameters are based on the primary structure of proteins and describe their hydrophobic nature from the point of view of composition. A more relevant parameter for protein–polymer interaction should describe the protein surface hydrophobicity, which is not only dependent on the primary structure of a protein, but also on its tertiary structure. The surface hydrophobicity of proteins has been previously measured and ranked by RPLC,[24,25] HIC,[10,26] protein partition between hydrophobically modified polyethylene glycol and dextran,[27] surfactant binding assays,[28] and fluorescence probe methods.[25,29] Indeed, the rank of protein surface hydrophobicity is very different from the hydrophobicity rank based on protein primary structure. For example, bovine serum albumin (BSA) is less hydrophobic than β-lactoglobulin (BLG) based on amino acid compositions,[22] but more hydrophobic than BLG according to surface hydrophobicity measurements.[10,26,28–30] Such differences in protein hydrophobicity ranking may even arise with the same type of measurements but different experimental designs. For example, the surface hydrophobicity of a protein probed by binding assay is likely to be a function of the ligand selected.[31,32]



R - Alkyl

SCHEME 1.

Synthetic polymers are much simpler than proteins with regard to composition and conformation, and their hydrophobicity may be adjusted by controlling composition. Thus, the hydrophobicity of water-soluble polymers may be modified either by direct copolymerization of hydrophobic and water-soluble monomers, or by postpolymerization functionalization.[33] Just as hydrophobic effects play a role in determining the tertiary structure of proteins, they also influence the conformation of flexible polyamphiphiles. As polymer hydrophobicity increases, the tendency of the polymers to form intra- and interpolymer aggregates becomes increasingly significant. Since such polymer conformational transitions may complicate the effect of polymer hydrophobicity on protein–polymer complexation, an understanding of the dependence of polymer conformation on its hydrophobicity becomes a prerequisite to the study of protein–polyelectrolyte hydrophobic interactions. In this regard, it is advantageous to use polymers with known dependence of conformation on polymer composition and solvent conditions. One type of such well-characterized polymers is a series of copolymers of maleic acid and vinyl alkyl ethers of various alkyl chain length (Scheme I). Dubin and Strauss[34] found that the conformation of these copolymers is jointly determined by the degree of ionization and hydrophobicity. The former is a function of solution pH and the latter is determined by the length of alkyl side chains.

Copolymers at low degree of ionization (i.e., at low pH) and with high hydrophobicity (i.e., with long alkyl side chain) tend to have a compact conformation and form intrapolymer micelles, in a so-called "hypercoil" conformation. Copolymers with opposite features exhibit more extended conformations. The critical solution conditions at which conformation transitions of these copolymers occur have been determined.

Since this work focuses on hydrophobic interactions, it is important that both the proteins and polymers under study exhibit various degrees of hydrophobicity. Therefore, the model system here is comprised of the proteins BSA (pI = 4.9) and BLG (pI = 5.2), and copolymers of maleic acid and vinyl alkyl ethers of different alkyl side chain length. Since these acidic proteins have similar pIs and all the polymers have the same ionizable group, these macromolecules—in neutral and basic solutions—provide for a range of hydrophobic interactions, while keeping the net repulsive electrostatic interaction relatively constant for different protein–polyelectrolyte pairs. The absence of strong electrostatic attraction reduces complications due to superimposable electrostatic and hydrophobic effects encountered in other studies. However, because the electrostatic effect due to local charge patches on protein surface is difficult to determine, hydrophobic contributions are best evaluated by comparing the binding of proteins to copolymers of different alkyl chain length.

EXPERIMENTAL

Materials and Sample Preparation

BSA (catalogue no. 100062) was purchased from Boehringer-Mannheim (Indianapolis, IN). β-Lactoglobulin A&B (catalogue no. L-2506) was purchased from Sigma (St. Louis, MO). A series of copolymers of maleic acid anhydride and vinyl alkyl (methyl, ethyl, butyl, hexyl, octyl, and dodecyl) ether were kindly provided by U. P. Strauss of Rutgers University. These copolymers[34] are rather polydisperse with average $M_w \approx$ 50k. High purity water of $R \approx 18$ (M$\Omega \cdot$ cm) generated by a Milli-Q® water purification system was used to make all solutions. Phosphate buffers were prepared from monobasic and dibasic salts of sodium phosphate obtained from J. T. Baker Chem. Co. (Phillipsburg, NJ).

Samples of proteins and copolymers solutions were mixed, based on volume, from freshly made stock solutions. Only BSA stock solution was filtered (0.45 μm Whatman syringe-filters, Clifton, NJ) before sample preparation. Concentrations of all protein stock solutions were determined spectrophotometrically at 280 nm using published extinction coefficients.[35] Each copolymer stock solution was made by first dissolving 15 mg copolymer in 9 mL salt-free basic aqueous solution (pH $\geq$ 10) under sonication; then, after complete dissolution and hydrolysis, adjusting the pH to neutrality and diluting the solution to the final volume with phosphate buffer of desired pH and ionic strength. Concentrations of these copolymer stock solutions were fixed at 1.0 g/L based on weight measurement. Binding of proteins to the copolymers were studied in two phosphate buffers: pH = 6.3 and $I = 0.05M$ (buffer 1) and pH = 8.7 and $I = 0.1M$ (buffer 2). The pHs of the buffers were selected to facilitate the study of the effect of the polymer conformation transition on hydrophobic interaction. The higher ionic strength for buffer 2 was chosen to suppress the electrostatic repulsion at high pH that could complicate the measurement of hydrophobic interactions.

Methods and Instrumentation

Capillary electrophoresis was carried out at 25 $\pm$ 0.2°C with a Beckman P/ACE 5500 (Fullerton, CA) equipped with a uv detector. A bare-fused silica capillary of i.d. = 50 μm from Restek (Bellefonte, PA) was used with a total length of 27 cm and an effective separation length (from inlet to detection window) of 20 cm. Separation voltage was in the range of 7–10 kV. Frontal analysis continuous capillary electrophoresis[36] was used to determine the free protein concentration in complex samples. This method has been shown[36] to exhibit less than 10% difference in duplicating binding isotherms with separate sample preparation and calibrations. Repeated experiments within samples used in this work showed that every point in the binding isotherms is reproducible within 3%, roughly the size of the data points in the figures.

Analysis of binding data depends on the selection of the model. Since the binding between the proteins and the copolymers is nonspecific, sharing the feature of large ligands binding to a linear lattice, the selected binding model must take into account the "overlapping binding site" effect.[37,38] However, our binding systems are further complicated by conformational changes of the copolymers upon binding and the possible linkage[39,40] of BLG oligomer equilibrium[41] to the binding. Therefore, although such binding systems are experimentally feasible to study, they are not readily fitted to any existing models. In the absence of a precise model for a complete binding

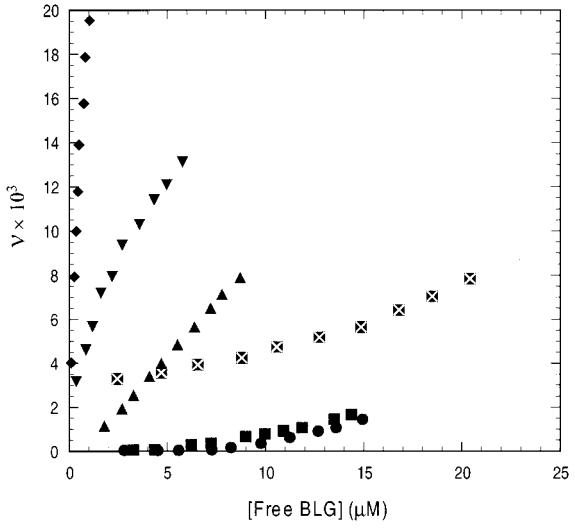


FIGURE 1 Binding isotherms for BLG complexing with various copolymers at 0.2 g/L in buffer 1 of pH = 6.3 and $I = 0.05M$. (■) Methyl, (●) ethyl, (▲) butyl, (◆) hexyl, (▼) octyl, and (⊠) dodecyl.

analysis, we focused our attention on determining intrinsic binding constants by using the McGhee–von Hippel noncooperative model[37,38] for overlapping binding sites systems, which is mathematically expressed as

$$\frac{v}{L} = K_b \cdot (1 - nv) \cdot \left(\frac{1 - nv}{1 - (n-1)v} \right)^{n-1} \qquad (1)$$

where L is the free protein concentration, K_b is the intrinsic binding constant (binding constant for the first protein binding to a protein-free copolymer), n is the average size of the binding sites, and v is the binding density defined as the average number of bound proteins per monomer unit (represented in Scheme I). The intrinsic binding constant is then extracted from the Scatchard plot (v/L vs v) by only fitting binding data obtained at low binding densities (the fitting was aided by Excel® 7.0 dynamically linked to DeltaGraph® 4.0), so that the effects that cause the deviation of the current system from the McGhee–von Hippel model can be minimized and the determination of K_b can be independent of any knowledge of n.[42] However, excluding data obtained at relatively high binding densities compromises the accuracy of fitting and a full understanding of the binding mechanism. Thus, the intrinsic binding constant so determined should only be regarded as estimates and semiquantitative.

RESULTS AND DISCUSSION

Binding Observed in Buffer 1 (pH = 6.3, $I = 0.05M$)

Binding isotherms for the complexation of BLG with copolymers of different alkyl side chains in buffer 1 are compared in Figure 1. The order of the binding affinities of different copolymers for BLG is hexyl > octyl > butyl ⩾ methyl and ethyl. Although BLG binds to the methyl copolymer slightly more than it binds to the ethyl copolymer, the difference is insignificant compared to the magnitude of BLG binding to the butyl copolymer. The binding isotherm for dodecyl copolymer has a different shape and intersects the isotherm for the butyl copolymer.

The effect of the alkyl side chain length on the hydrophobic interaction between copolymers and proteins is seen to have two important features. First, a minimum alkyl side chain length of 3–4 carbon atoms is required for hydrophobic interactions to matter. This observation is reminiscent of Tanford's finding,[43] based on the free energy of transfer of hydrocarbons from aqueous solution to apolar solvent, that the first and possibly the second carbon atom in an alkyl chain attached to a polar group have only minute contribution to the hydrophobicity of the molecule. However, such a minimum chain length for significant hydrophobic interactions is shorter than the previously reported six carbon atoms for alkyl chains immobilized on agaroses used in HIC.[10,12] The difference is probably due to the flexibility of the alkyl chain on a free polyelectrolyte that can adapt to the size and the shape of a protein, thus facilitating hydrophobic interactions. This is in agreement with the consideration that the effective hydrophobicity of a hydrocarbon chain also depends on its flexibility.[44]

The second observation is that the copolymer conformation affects the ability of its alkyl side chains to interact with proteins. The alkyl side chains of copolymers in the random coil conformation exhibit stronger hydrophobic interaction with BLG than the ones in the hypercoil conformation. Previous potentiometric titration results[34] show that at pH = 6.3 (buffer 1), methyl, ethyl, and butyl copolymers are in the random coil conformation, while the hexyl copolymer is in transition between hypercoil and random coil, and copolymers with alkyl chain length longer than octyl are hypercoiled. The effect of increasing ionic strength, which shifts the hypercoil → random coil transition to a slightly higher pH, is considered to be a secondary factor. Since the state of most of the copolymers under study comprises both random coil and hypercoil moieties, the observed BLG binding

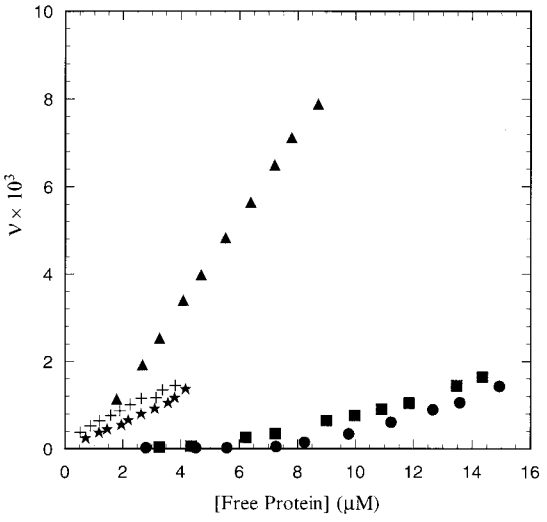


FIGURE 2 Comparison of binding isotherms for BSA and BLG complexing with various copolymers at 0.2 g/L. (+) BSA/methyl, (⋆) BSA/ethyl, (■) BLG/methyl, (●) BLG/ethyl, and (▲) BLG/butyl buffer 1 of pH = 6.3 and $I = 0.05M$.

affinity order in Figure 1 can be explained based on the assumption that the protein only interacts with unmicellized alkyl side chains. Without a detailed knowledge of the dynamic nature of the copolymer conformation in the time scale of the binding process, one needs to consider two possibilities. One is that the unmicellized alkyl side chains, regardless of number, may be relatively static. Another scenario is that the conformation of the copolymer is rather dynamic and the alkyl side chains are in fast equilibrium between random coil and hypercoil states. Since a static picture of alkyl side chain conformation only leads to a steady increase in the intrinsic binding constant and the slope of the binding isotherm as the alkyl side chain length increases, which is clearly in conflict with the experimental observation, the conformation of alkyl side chain is likely to be dynamic, and proteins can interact with any alkyl side chain by overcoming the free energy change associated with the hypercoil → random coil transition at a particular solvent condition. The apparent binding constants extracted from these isotherms can thus only be used to calculate the net free energy change due to the binding and the concomitant conformation transitions of the alkyl side chains at the binding site.

The binding isotherms for BSA with methyl and ethyl copolymers in buffer 1 are compared with those for BLG in Figure 2. Data for BSA binding to other copolymers are not available due to the difficulty in electrophoretically separating free BSA from its complexes under the current experimental conditions. As seen in Figure 2, BSA shows stronger binding for methyl and ethyl copolymers than BLG does. However, compared to the magnitude of binding exhibited by BLG with the butyl copolymer, binding of BSA to the methyl and ethyl copolymers is still small, and the difference in binding density between BSA/methyl and BSA/ethyl is even smaller. Nevertheless, duplicate experiments confirm that both BSA and BLG show consistently larger binding with the methyl relative to the ethyl copolymer. Although such a phenomenon is counterintuitive, the results imply that the observed binding was not due to hydrophobic interaction between the proteins and the methyl or the ethyl side chains of the copolymers.

Binding Observed in Buffer 2 (pH = 8.7, $I = 0.1M$

At pH = 8.7, all copolymers, except for the dodecyl copolymer, are in the random coil conformation. BLG only binds to copolymers with alkyl side chains longer than butyl. The negligible binding of BLG to the butyl copolymer (not shown) is presumably due to increased electrostatic repulsion between negatively charged proteins and copolymer at this high pH. The binding isotherms in Figure 3 show that the difference between octyl and hexyl copolymers is smaller than for buffer 1 (Figure 1). As is the case for buffer 1, the dodecyl copolymer shows the least binding.

Changes in pH and ionic strength affect not only the copolymer conformation and protein surface hydrophobicity, but also the charges on proteins and copolymers. According to the pH titration results,[45,46]

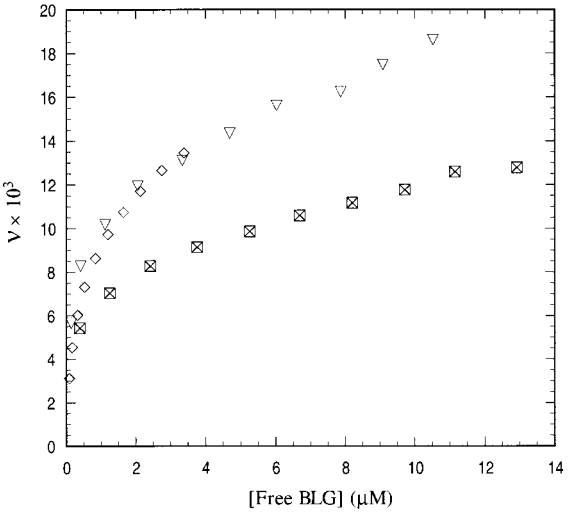


FIGURE 3 Binding isotherms for BLG complexing with various copolymers at 0.2 g/L in buffer 2 of pH = 8.7 and $I = 0.1M$. (◇) Hexyl, (▽) octyl, and (⊠) dodecyl.

Table I Intrinsic Binding Constants (K_b) for BLG with Various Copolymers

	$K_b \times 10^{-4}$; M^{-1}	
Copolymers	buffer 1 pH = 6.3; $I = 0.05M$	buffer 2 pH = 8.7; $I = 0.1M$
Hexyl	$K_b = 7.5$; $n = 30$	$K_b = 6.4$; $n = 60$
Octyl	$K_b = 1.0$; $n = 40$	$K_b = 10$; $n = 55$
Dodecyl	$K_b = 0.5$; $n = 150$	$K_b = 3.2$; $n = 68$

the net charges on BSA and BLG are −11.5 and −9.3, respectively, in buffer 1, and −28.4 and −20.7, respectively, in buffer 2. Therefore, comparison of binding isotherms at different pHs involves consideration of changes in both hydrophobic and electrostatic interactions. At the higher pH of buffer 2, the increased negative charge for BLG and the copolymers not only leads to increased electrostatic repulsion between BLG and copolymers, which tends to decrease the extent of binding, but also to increased repulsion among adjacent bound proteins. The latter may be responsible for the more prominent anticooperativity manifested by the upward curvature in all the Scatchard plots in buffer 2. On the other hand, an increase in buffer pH favors the random coil conformation of the copolymer, which tends to enhance hydrophobic interactions between copolymers and proteins by making alkyl groups more accessible. The observed binding is the net result of the two competing processes. The effects of pH on binding can be discussed semiquantitatively based on values of intrinsic binding constants (Table I) estimated by fitting the data at low binding density in the two buffers. The theoretical curves, corresponding to the fitted parameters listed in Table I, are shown along with the experimental data in Figures 4–6. These binding isotherms and Scatchard plots allow a visual evaluation of the goodness of fit. The experimental data at high protein concentration are seen to deviate systematically in the direction of stronger binding from the theoretical curves obtained by fitting to the low binding density results. The inability of the overlapping binding site model to fit data in the high binding density regime probably arises from changes in polymer conformation upon binding (see next section), which may make more sites available for binding. Such effects are quite difficult to model in any definitive way.

All the values of n in Table I are considerably larger than 11 monomer units, a calculated size based on the hydrodynamic diameter of BLG (5.4 nm) and the one-dimensional size of monomer units (5.0 Å). With the exception of the BLG/hexyl copolymer in buffer 1, the fitted n values are also greater than 34 monomer units, the length required for the copolymers to "wrap around" the protein. Two factors may contribute to these large apparent binding site sizes. First, the compact conformation of the copolymers (due to intrapolymer micellization) may make it impossible for a given contour length of polymer to encompass many proteins: this effect could account for the monotonic increase of binding site sizes from the hexyl copolymer to the dodecyl copolymer in buffer 1. A second effect is the anticooperativity in binding due to electrostatic repulsion between adjacent bound proteins, which can be imbedded into the fitted size of the binding site. This factor may explain why apparent binding site sizes are larger in buffer 2 than in buffer 1 (for hexyl and octyl copolymers) despite the fact that copolymers are more extended in buffer 2 than in buffer 1. Complex variations in binding affinity with pH and ionic strength arise because of such manifold effects on hydrophobic and electro-

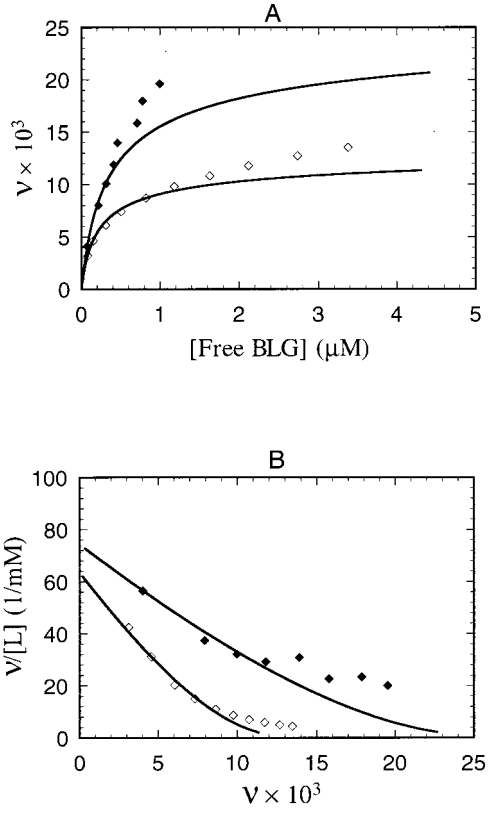


FIGURE 4 Binding of BLG to hexyl copolymer (0.2 g/L). Symbols are experimental data in buffer 1 (◆) and buffer 2 (◇). Lines are fitted theoretical curves (parameter values in Table I) weighted toward low binding density data. (A) Binding isotherms. (B) Scatchard plots ([L] is free BLG concentration).

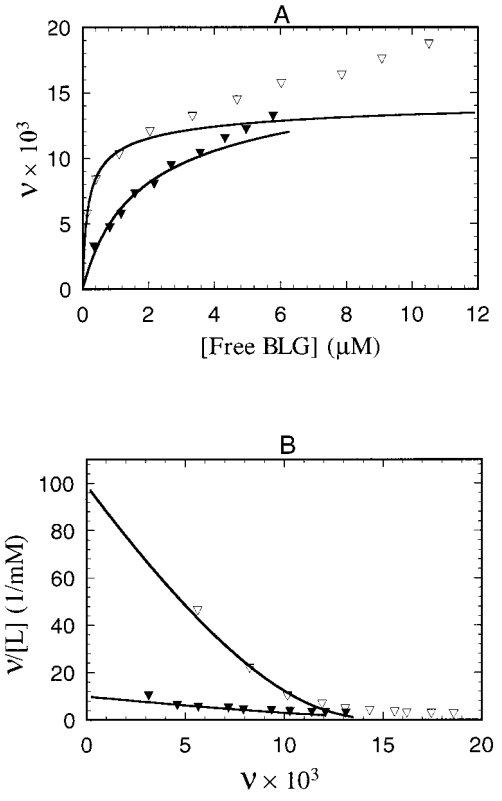


FIGURE 5 Binding of BLG to octyl copolymer (0.2 g/L). Symbols are experimental data in buffer 1 (▼) and buffer 2 (▽). Lines are fitted theoretical curves (parameter values in Table I) weighted toward low binding density data. (A) Binding isotherms. (B) Scatchard plots ([*L*] is free BLG concentration).

static interactions among the protein and the copolymers.

According to Figure 4, the binding of BLG to hexyl copolymer is weaker in buffer 2 than in buffer 1. The possible gain in BLG–hexyl copolymer hydrophobic interaction due to the breakdown of intrapolymer micelles with increasing pH is not enough to offset the increased electrostatic repulsion, resulting in a drop in K_b as shown in Table I. On the other hand, stronger binding was observed for the octyl copolymer at higher pH (Figure 5), as reflected in a tenfold increase in K_b shown in Table I. This substantial increase in binding indicates that hydrophobic accessibility outweighs the negative contribution from increased electrostatic repulsion: the octyl copolymer is fully hypercoiled in buffer 1 and essentially a random coil in buffer 2. For the dodecyl copolymer, higher pH also leads to stronger binding (Figure 6); however, only a sixfold increase in K_b is seen (Table I), since this copolymer is not completely in the random coil conformation at higher pH.

Molecular Models of Conformation Change of Copolymers Upon Protein Binding

The competition between intrapolymer micellization and protein binding suggests that some degree of polymer conformation change might be induced by the binding of proteins. Figure 7A, for example, schematically represents a complete disruption of intrapolymer micelles upon binding of protein, which may occur if the free energy of binding completely overcomes the free energy of disruption of intrapolymer micelles. The binding of BLG to hexyl copolymer in buffer 1 resembles this situation. In the scenario of Figure 7B, the binding of protein induces only a partial breakdown of intrapolymer micelles, because the binding free energy fails to overcome the energy barrier for a complete polymer conformation change. Binding of BLG to octyl copolymer in buffer 1 may represent this case. Figure 7C depicts the case in which the binding free energy is small compared with

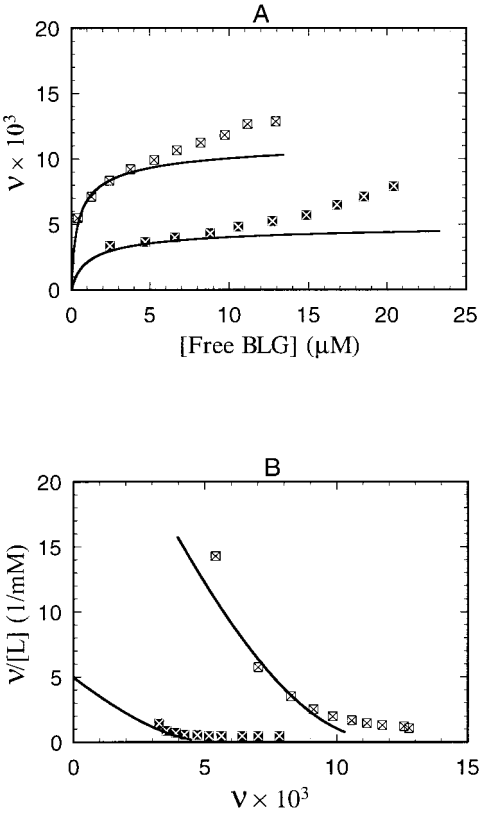


FIGURE 6 Binding of BLG to dodecyl copolymer (0.2 g/L). Symbols are experimental data in buffer 1 (⊠) and buffer 2 (⊠). Lines are fitted theoretical curves (parameter values in Table I) weighted toward low binding density data. (A) Binding isotherms. (B) Scatchard plots ([*L*] is free BLG concentration).

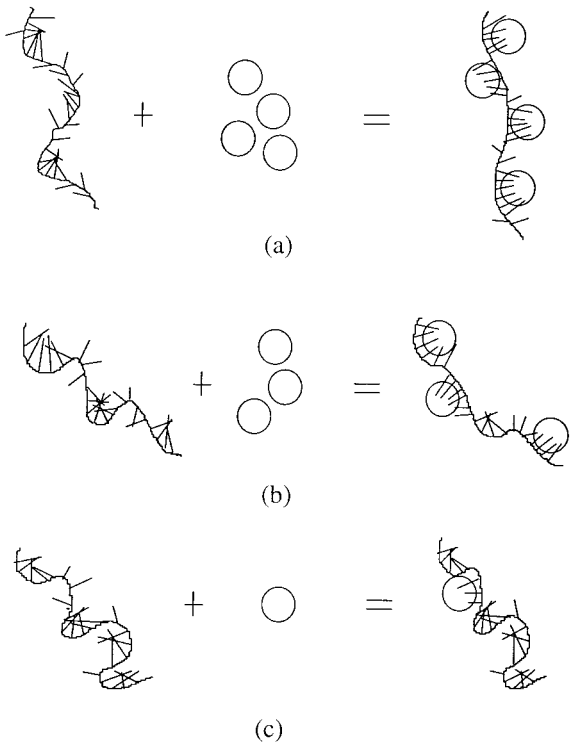


FIGURE 7 Models of polymer conformation changes upon the binding of proteins. (A) Complete, (B) partial, and (C) minimal disruption of intrapolymer micelle formation by bound proteins.

that of intrapolymer micelle formation, and proteins can only interact with the small number of alkyl chains not able to participate in intrapolymer micelle formation due to geometric restrictions. The binding of BLG to dodecyl copolymer in buffer 1 resembles this situation.

Free Energy of Hydrophobic Interaction

The hydrophobic nature of the binding would be clarified by thermodynamic analysis. However, several difficulties complicate the determination of the appropriate free energies. Since the binding process is frequently accompanied by the polymer conformational transition, it is necessary to subtract the free energy of the polymer conformation change from the free energy measured for the overall process in order to calculate the free energy associated with binding *per se.* This requires a knowledge of both the size of the protein binding site on the copolymer and the size of an intrapolymer micellar cooperative unit. Estimates of the former can be made from fitting the isotherms to a model, while values for the size of the copolymer cooperative unit have been obtained from previous potentiometric studies.[34] To determine the intrinsic hydrophobic binding free energies, one also needs to subtract the free energy contributions from the repulsive electrostatic forces. Most problematically, one needs to know the exact number of alkyl side chains interacting with the protein at the binding site.

Although the present results do not lead to quantitative parameters for the interaction of a protein with an exposed hydrophobic site on the polymer chain, they clearly demonstrate the role of accessibility in intermacromolecular hydrophobic interactions. As noted above, a minimum length of six carbons is required to observe hydrophobic interactions between proteins and alkyl groups attached to chromatographic surfaces, in contrast to the four carbons observed here for amphiphilic polyelectrolytes. The difference is attributable to the flexibility of the latter. It may be generally stated that hydrophobic interactions take place only when the regions of perturbed water structure around the two hydrophobic moieties overlap. Although the dimensions of these regions may be large for large apolar domains, e.g., hydrophobically modified mica planes, they are likely to be small for isolated alkyl or phenyl groups, particularly ones surrounded by polar functional groups. Thus, favorable interactions may require intimate contact of the two hydrophobes. If both hydrophobes are constituents of macromolecules, this requires either geometric complementarity, as in the case of relatively rigid macromolecules such as proteins, or chain mobility, as in the case of "random coil" polymers. In the present situation, both the accessibility of the protein's hydrophobic regions and the flexibility of the polyelectrolyte chain are factors that facilitate hydrophobic association.

On a practical level, the current findings may support the design of chromatographic stationary phases. The copolymer conformation is seen to have a profound influence on the extent of protein–copolymer interactions. Since such interactions are hydrophobic in nature, the polymer conformation determines its "effective hydrophobicity" to a certain extent. Therefore, the dependence of polymer conformation on solution pH implies that these copolymers possess effective hydrophobicities adjustable via pH. Such a property could be desirable for the stationary phase of RPLC and HIC, because the retention of a solute can then be adjusted by a pH gradient, which is fundamentally different from superimposing a pH-sensitive ionic interaction on top of a hydrophobic interaction as proposed before.[47] The resultant use of aqueous solutions instead of gradient elution using organic modifiers could then minimize the use of organic

modifiers in separation and reduce environmental hazards.

CONCLUSIONS

Hydrophobic interactions between proteins and amphiphilic copolymers have been quantitatively evaluated by systematically varying the length of the copolymer alkyl side chain. A minimum alkyl side chain length of 3–4 carbons is required for significant hydrophobic interactions. As the length of the alkyl side chain increases, the tendency of the copolymers to form intrapolymer micelles and to bind with proteins both increase. Competition between the two processes explains the order of binding affinity observed at different pHs.

We thank Prof. U. P. Strauss for supplying the copolymer samples. This work was supported by Grant DMR 9619722 from the National Science Foundation. J.Y.G. acknowledges a fellowship from Purdue Research Foundation.

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